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Effects of rain events on *Cryptosporidium* spp. levels in commercial shellfish zones in the Hillsborough River, Prince Edward Island, Canada

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ABSTRACT

A longitudinal study was conducted during the 2014 spring eastern oyster harvesting season in Prince Edward Island. The concentrations of *Cryptosporidium* oocysts were assessed in seawater and oyster samples taken from three commercial shellfish zones (Approved, Restricted, and Prohibited) one day before a rain event (of at least 15 mm), and on days 1, 3, and 7 after the rain event. Two rain events were recorded during the study period. The first event (rain event 1) was characterized by precipitation of 15 mm over 14 h (low intensity) and the second event had 40 mm in 14 h (high intensity). Oocysts in 20 L of seawater ranged from 0 to10, 0 to 7, and 1 to 15 in Approved, Restricted, and Prohibited zones, respectively, and in oysters, ranged from 0 to 30, 0 to 48, and 0 to 25 in Approved, Restricted, and Prohibited zones, respectively. A significant increase in *Cryptosporidium* spp. oocyst counts was observed in seawater samples after an intense rain event. Oocyst counts in seawater were almost two times higher after a high intensity rain event than after a low intensity event, but no effect was observed in oysters. However, positive samples (seawater and oysters) were still present seven days after a rain event. These findings suggest that authorities should consider monitoring for *Cryptosporidium* spp. in shellfish and their associated waters, especially after heavy rain events.

1. Introduction

The protozoan parasite *Cryptosporidium* spp. can cause gastrointestinal disease (King and Monis, 2006) in people, including mortality of immunocompromised people, due to the lack of a fully effective anti-cryptosporidial therapy (Dawson, 2005). *Cryptosporidium* spp. is considered an emerging pathogen (Baishanbo et al., 2005), and is one of the most significant diarrheal pathogens affecting people worldwide (Shirley et al., 2012). Transmission of the parasites is via environmentally-resistant oocysts, which are infectious when excreted in the feces (Baishanbo et al., 2005). Oocysts are transmissible to humans and other animals via direct contact with contaminated fecal material or by contact with, or consumption of, contaminated sources, such as water and food (Dixon et al., 2011).

Commercial oysters, worldwide, have been associated with the presence of infectious oocysts (Graczyk et al., 2007). The two main species of *Cryptosporidium* reported in oysters are *C. parvum* and *C. hominis* (Robertson, 2007). Oysters feed by filtering large volumes of seawater and, during this process, they can accumulate and concentrate *Cryptosporidium* oocysts (Iwamoto et al., 2010; Willis et al.,

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2013), which are dispersed in seawater where oysters are harvested. It has been documented that, although with a relatively low infection dose (Messner et al., 2001), the accumulation of this parasite in shellfish, if consumed raw, poses a health risk to humans (Robertson, 2007). However, the association of disease with the consumption of raw oysters with cryptosporidiosis has not been documented. This could be due to the long duration between exposure and symptoms of illness, which could last up to 15 days (average 7 days) (Cacciò and Putignani, 2014), and that most people do not visit a doctor for an infectious gastrointestinal illness (IGI). It has been reported that for every IGI reported, between 310 and 350, on average, are not reported (Majowicz et al., 2005; MacDougall et al., 2007).

In Canada, during the spring harvesting season, oysters are only harvested in Restricted zones, and shellfish must be subject to a decontamination plan (e.g., depuration, natural relaying, container relaying, or canning) which has been accepted by the shellfish control authority before they can be sold commercially (CFIA, 2015). Decontamination plans and lengths are established according to coliforms counts and inorganic sources levels (CFIA, 2015), but they completely ignore the presence of parasites such as *Cryptosporidium* spp. (Willis et al., 2013). It has been shown that *Cryptosporidium* oocysts can remain infective for >14 days in oysters (Gómez-Couso, 2003; Willis et al., 2013), longer than the minimum duration of the relay process in Canada (Rees et al., 2010).

The level of *Cryptosporidium* spp., in seawater is affected by different environmental factors, such as overboard sewage discharge into harvest areas; sewage runoff after heavy rains or flooding (Iwamoto et al., 2010); agricultural activities, such as manure storage and application to arable land; discharge of contaminated water; pasturing of livestock near water sources; and disposal of fecal-contaminated waste from abattoirs and wastewater treatment facilities (Schijven et al., 2004).

In the Hillsborough River of Prince Edward Island (PEI), Canada, an area that has a mixture of urban and agricultural land uses, the oyster fishery is regulated into three zones: Approved, Restricted, and Prohibited. In Canada, an Approved zone is an area is that is not contaminated with faecal material, pathogenic micro-organisms, poisonous or deleterious substances, to the extent that consumption of the shellfish might be hazardous. In an Approved zone, the median faecal coliform Most Probable Number (MPN) of the water does not exceed 14/100 mL, and not >10% of the samples exceed a faecal coliform MPN of 43/100 mL, for a five-tube decimal dilution test. (CFIA, 2015). A Restricted zone is a contaminated area, or has the potential to become contaminated, provided the area is not contaminated to the extent where it would be classified as Prohibited. The median MPN of the water exceeds 14/100 mL, and/or >10% of the samples exceed a faecal coliform MPN of 43/100 mL, for a five-tube decimal dilution test. Finally, oysters from Prohibited zones cannot be fished for consumption, as these zones contain all the wastewater release sites as, for example, around the municipalities of Charlottetown and Stratford, and the Queen Elizabeth Hospital (Prince Edward Island).

Currently, during heavy rainfall, the municipal wastewater treatment plant can overflow and result in the release of untreated sewage. In addition, animal effluent from agricultural land, which potentially contains pathogenic parasites, can also be released into the aquatic environment during a run-off event; both factors can potentially increase the levels of *Cryptosporidium* spp. in the water and, hence, in the oysters.

The objectives of this study were to measure the levels of *Cryptosporidium* oocysts in seawater and oysters from Approved, Restricted, and Prohibited shellfish harvest zones in the Hillsborough River of Prince Edward Island during the spring season, before and after a rain event.

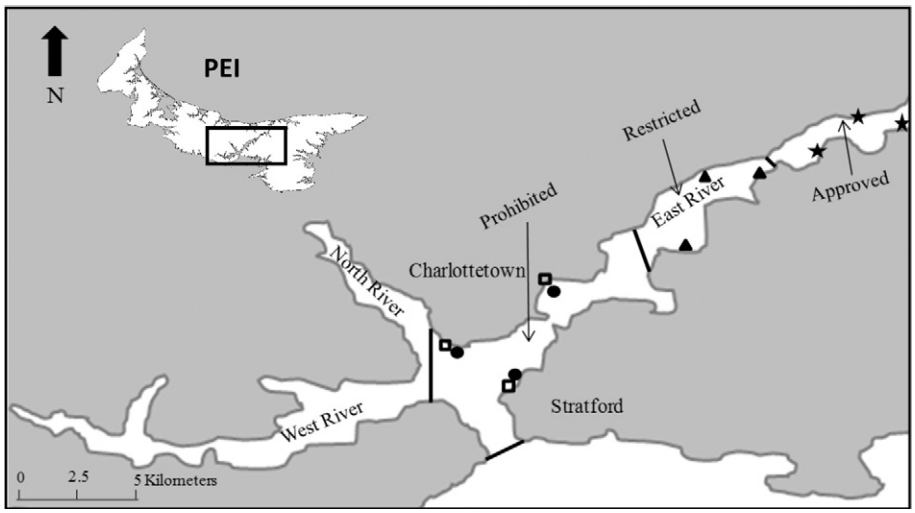

Fig. 1. Sites for sampling seawater and oysters in the Hillsborough (East) River. Circles, triangles, and stars indicate sites in the Prohibited, Restricted, and Approved oyster fishery zones, respectively, as managed by the Department of Fisheries and Oceans. Zonal boundaries are indicated with the black lines. Squares mark the main effluent outflows for the municipalities of Charlottetown and Stratford.

2. Materials and methods

2.1. Water sample collection and processing

A longitudinal sampling design was used to monitor the level of *Cryptosporidium* oocyst contamination in two separate periods during the spring and summer of 2014 in PEI. In each oyster fishery zone—Approved, Restricted, and Prohibited (Fig. 1)—three water samples were taken one day before a day of precipitation (≥15 mm/h forecast prediction by Environment Canada) and one, three, and seven days after the rain event ended in the Hillsborough River System (Fig. 1) at three different sampling points in each zone.

Sampling sites were selected close to locations with the potential to be sources of contamination (hospital, sewage, agricultural land, etc.). Each sample consisted of 20 L of water collected as close as possible to the bottom of the riverbed and oyster beds. Samples were stored in 20 L bottles at 4 °C prior to processing, which occurred between 2 and 24 h post-collection.

Water samples were filtered through a Filta-Max® (IDEXX, Westbrook, ME, USA) foam filter system according to the US-EPA method 1623 (USEPA, 2005) and the manufacturer's instructions. Once filtered, the Filta-Max® filters were placed into clean plastic bags, sealed and leveled, and stored at 4 °C in the laboratory for <24 h until further processing. Filtered samples were processed for *Cryptosporidium* spp. isolation and enumeration according to US-EPA method 1623 (USEPA, 2005), using the Filta-Max® wash station procedure for elution of oocysts. *Cryptosporidium* oocysts were isolated from ≤0.5 mL water pellets by immunomagnetic separation, using anti-*Cryptosporidium* coated magnetic beads (Dynal, Lake Success, NY, USA) according to manufacturer's instructions. Parasites were enumerated by epifluorescence microscopy (Leica DM 2500, Leica Microsystems, Wetzlar, Germany). A sample was considered positive if the oocysts fulfilled the morphological criteria defined in the US-EPA 1623 protocol (USEPA, 2005).

The number of oocysts detected per 20 L of seawater was calculated according to a previously described method (Budu-Amoako et al., 2012; Farias et al., 2002), as follows:

i. Number of oocysts in pellet = Number of oocysts of a 20 μL drop × total ml of the pellet/volume;
ii. Number of oocysts per 20 L = Number of oocysts in pellet times liters filtered.

2.2. Oyster sampling and processing

From nine to 11 oysters were collected in the Hillsborough River System (Fig. 1) at three different sampling points one day before a day of precipitation (≥15 mm/h forecast prediction by Environment Canada) and one, three, and seven days after the rain event ended in each harvesting zone (Approved, Restricted and Prohibited). Samples were stored at 4 °C prior to processing, which occurred between 24 and 48 h post collection. Individual oysters (tissue and hemolymph) were excised from their shells by cutting the anterior and posterior adductor muscles with an oyster shucking knife, carefully poured into 50 mL tubes, and homogenized for 1 min using an Omni tissue homogenizer (OMNI International, Kennesaw, GA). Homogenized samples were then incubated at 35 °C with 15 mL of pepsin-HCl solution for 75 min, and removed from the incubator every 25 min and vortexed for 20 s. Samples were centrifuged at 900 × *g* for 5 min, and the resulting pellet was subsequently washed and re-centrifuged with water, phosphate buffered saline (PBS) eluting fluid (pH 7.4), and again with water. The final pellet was suspended in 50 μL of PBS. Pepsin–HCl digestion solution and PBS eluting fluid were prepared according to Robertson and Gjerde (2008).

Subsamples of 50 μL of oyster homogenate were air-dried to fluorescence microscopy slides (Waterborne Inc., New Orleans, LA, USA). Samples were stained with 50 μL of *Cryptosporidium*-specific fluorescein isothiocyanate labeled monoclonal antibody solution (Crypt-a-glo™, Waterborne Inc., New Orleans, LA, USA). Slides were incubated in a humid chamber for 40 min, briefly rinsed with PBS, and allowed to air-dry overnight in a dark slide box. Fluorescent antibody mounting fluid (AquaPoly-mount, Polysciences, Warrington, PA, USA) was used to adhere coverslips, and oocysts were detected and enumerated at 600× magnifications using an epifluorescence microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany). The number of oocysts per sample was defined as the number of oocysts per slide well divided by the volume of the sample analyzed, and multiplied by the total μL of sample.

2.3. Data analysis

The numbers of oocysts in water and oyster samples were estimated by 20 L of seawater and by oyster, respectively. Summary statistics by time from rain event, zone, and rain event were computed for each sample type. The association between zone (Approved, Restricted, and Prohibited), time (days of sampling), rain event, and the number of oocysts was estimated using a repeated measures mixed effects Poisson regression in the statistical package Stata V.14 (StataCorp, 2015).

Counts were used as the dependent variable, and the following predictors were included: rain event (low and high intensity); time (1, 3, and 7 days from rain event); pre-rain parasite counts; and zone (Approved, Restricted, and Prohibited). For the water sample model, the sampling site was used as random effect and the repeated measures within each site was modelled using a random slope between site and time. On the other hand, for the oyster model, given that the repeated measures were at the site level and there were several oysters sampled at each sampling time, we created a level of site * time and used this as random effect below site. Predicted concentrations of the *Cryptosporidium* spp. oocysts were obtained from each model. Evaluation of the fit and residual analysis was done using standard approaches (Dohoo et al., 2009).

3. Results

Two rain events occurred during the study period. The first event took place on June 6th, 2014, and was characterized by precipitation of approximately 15 mm over 14 h (low intensity event). The second event started on August 6th, 2014, and approximately 40 mm of rain fell in 14 h (high intensity event). Data for the two events was obtained from records published on the Environment Canada website (http://climate.weather.gc.ca/).

A total of 72 water samples were collected during the two rain events across the three zones and at three sampling points per zone. *Cryptosporidium* spp. oocysts were present in 75%, 100%, and 58% of the samples from the Approved, Restricted, and Prohibited zones, respectively. The mean number of oocysts in 20 L of seawater ranged from 0 to 10, 0 to 7, and 1 to 15 in Approved, Restricted, and Prohibited zones, respectively.

Fig. 2 shows the *Cryptosporidium* oocysts' mean, minimum, and maximum counts in water samples from Approved, Restricted, and Prohibited zones in Hillsborough River, for the two rain events. On average, the level of parasites was highest in the Restricted zone, followed by the Approved zone, while the Prohibited zone showed the lowest level of parasites, except at day 7 post rain event (Fig. 2 A–B).

The descriptive analysis indicated that the level of parasites was affected by the intensity of the rain event and shellfish area. The results from the mixed effects random slopes Poisson model are presented in Table 1, and indicate that the effect of time on the level of parasites depended on the zone (P-value = 0.037) and the intensity of the rain event (P-value = 0.029). In addition, there was significant variability across the 18 sampling sites.

When the rain event was of lower intensity and volume (<15 mm over 14 h), without intensive run-off (Fig. 2-A), the level of oocysts tended to be similar across all sampling times. However, it depended on the zone and time after rain event (Fig. 3A). The Restricted zone had the highest counts and remained stable during the three sampling times. On the other hand, the counts in the Prohibited zone increased, while the opposite was observed in the Approved zone. When a larger volume of precipitation occurred, a higher level of contamination with a more marked temporal pattern (Figs. 2B and 3B) was observed. The Restricted zone had the highest counts, with the largest values, 24 h after a rain event. On the other hand, the Approved zone presented a decreasing pattern in counts, while the contamination level for the Prohibited zone was constant, with a slight increasing trend. The model residuals did not indicate any particular outlying observations, and random effects were approximately normally distributed.

The overall mean number of oocysts per oyster across all sampling points and times ranged from 0 to 30, 0 to 48, and 0 to 25 in Approved, Restricted, and Prohibited zones, respectively. The contamination levels showed a pattern similar to the water samples. The low-intensity event tended to produce a lower contamination, and remained almost constant during the entire sampling period (Fig. 4-A), while the high-intensity event produced a higher contamination in oysters from the Restricted zone, which also showed a more pronounced temporal pattern. Higher counts were observed 24 h after the rain event ended, and declined until the end of the study (Fig. 4-B).The count regression model for oyster counts did not detect any statistical significance for both main effects and interaction between time and zone for all the predictors assessed in the model.

4. Discussion

The number of *Cryptosporidium* spp. oocysts present in seawater was more influenced by both the harvesting zone and the characteristics of the rain event than by the counts observed in oysters. However, none of the observed counts were associated

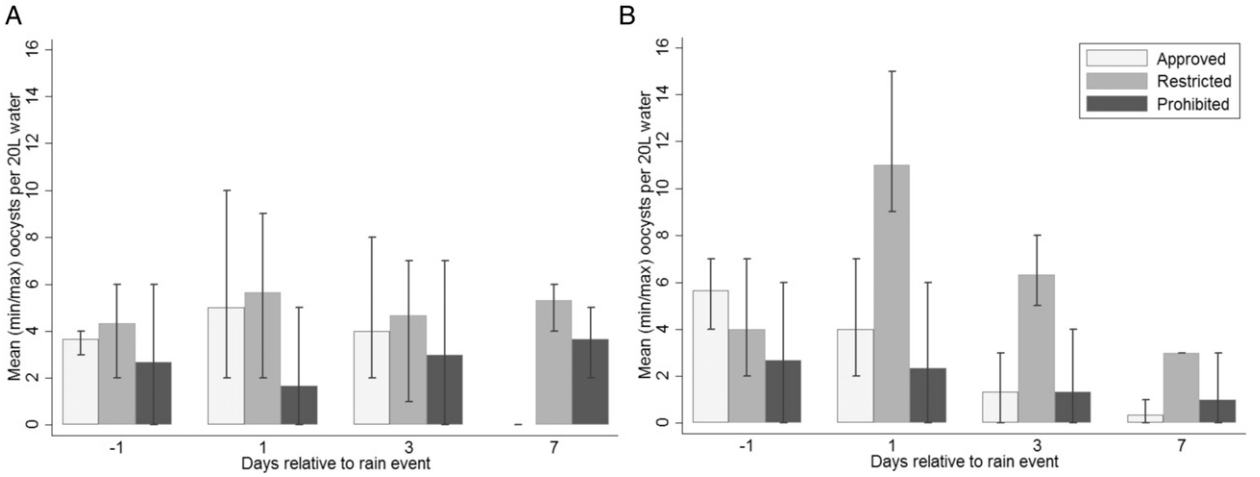

Fig. 2. Mean (bar), minimum and maximum (lines) *Cryptosporidium* spp. oocyst counts in seawater samples 14 h in Approved, Restricted, and Prohibited zones in the Hillsborough (East) River on Prince Edward Island, in Summer 2014 for (A) the low intensity rain event - at 15 mm over 14 h and (B) the high intensity rain event - 40 mm in 14 h.

Table 1
Mixed effect Poisson regression model for total oocyst counts in water samples collected three times from 18 sampling sites from three harvesting zones in the Hillsborough (East) River during the summer of 2014 in Prince Edward Island.

Predictor	Coefficient	*P*-value	95% C.I.
Pre-rain counts			
Linear term	0.857	0.012	0.188; 1.527
Quadratic term	−0.102	0.015	−0.186; −0.120
Sampling time		0.028	
1 day post rain	Reference		
3 days post rain	−0.234	0.502	−0.919; 0.450
7 days post rain	−2.772	0.008	−4.814; −0.729
Zone		0.019	
Approved	Reference		
Restricted	0.837	0.041	0.034; 1.640
Prohibited	−0.370	0.483	−1.403; 0.664
Zone * Sampling time	Shown in Fig. 3	0.028	
Rain event			
Low intensity	Reference		
High intensity	0.413	0.256	−0.230; 1.126
Rain event * Sampling time	Shown in Fig. 3	0.029	
Random effects	Variance	S.E.	95% C.I.
Sampling site			
Intercept	0.339	0.234	0.088; 1.308
Slope (sampling time)	0.002	0.004	0.00003; 0.121

with the risk of contamination according to the pre-defined areas, based on coliform counts by Canadian regulations (CFIA, 2015). Few studies have been conducted in estuaries with similar environmental and topological characteristics as in our study. Studies conducted in much larger populated areas showed much higher oocyst counts, which might reflect the size of the human populations close to the study areas (Farias et al., 2002). In a study conducted in southern Italy, five of 21 wastewater samples (treated water) and eight of 21 samples taken from downstream water flowing from the treatment plant into a lagoon were positive for *Cryptosporidium* spp. However, no oocysts were detected in oysters harvested from the lagoon (Giangaspero et al., 2009). These farms were located approximately 10 km away from the water sampling sites. The number of oocysts per liter ranged from 0 to 5800, but most of the samples were negative throughout the 12-month sampling period. However, the large variation in recovery efficiencies of *Cryptosporidium* spp. oocysts in water samples could also impact the counts reported in these studies (Ongerth, 2013).The number of oocysts found in the oysters in our study is lower than previously reported figures in the eastern U.S., where Graczyk et al. (2006) reported mean values of total and viable *C. parvum* oocysts of 42.1 (range: 5–105) and 28 (range: 5–106), respectively.

Intensity of rain event was more associated with counts in the oysters than in seawater samples. Oysters had, on average, almost twice as many oocysts when exposed to a high-intensity event as compared to a low-intensity event. However, no difference was observed in seawater samples. This finding could be due to the fact that oysters retain more of the oocysts (Gómez-Couso, 2003). Bivalve molluscs are recognized as good bioindicators of contamination particulates, including oocysts (Fayer et al., 1998).

Oocysts in the water, even after a short period of rainfall, can be diluted, depending on currents, tidal flows, and other bathymetric characteristics, and affected by other weather conditions, limiting the use of water samples to estimate risk of exposure to this parasite (Rose et al., 1997). Tidal effect might have been a factor that influenced the water contamination levels in different zones.

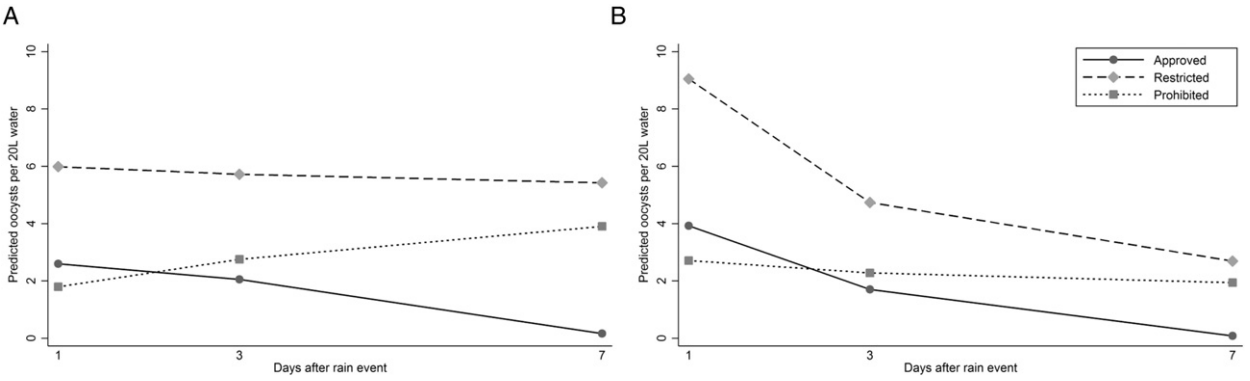

Fig. 3. Predicted mean counts of *Cryptosporidium* spp. oocysts in water samples for the Approved, Restricted, and Prohibited zones, after (A) the low intensity and (B) the high intensity rain events.

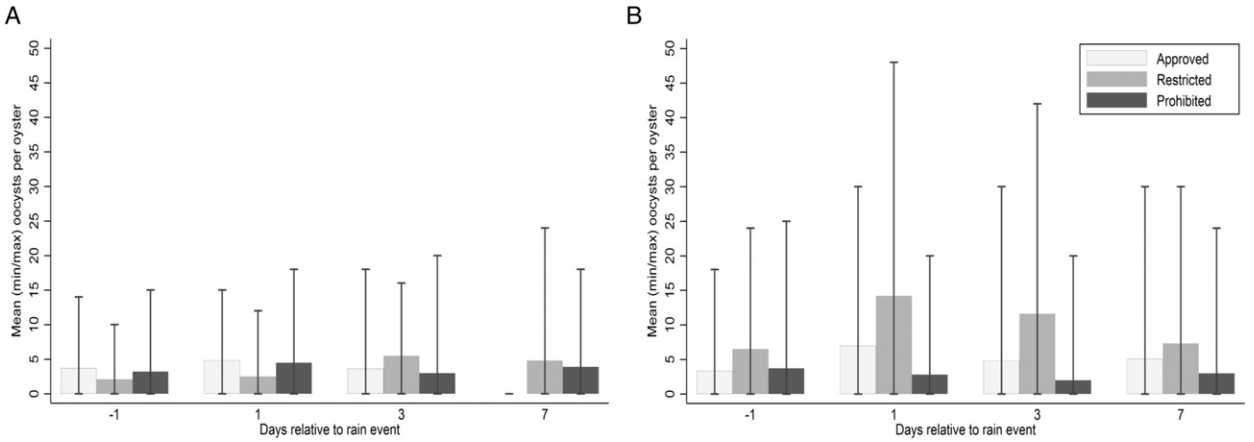

Fig. 4. Mean (bar), minimum and maximum (lines) *Cryptosporidium* spp. oocyst counts in oysters in Approved, Restricted, and Prohibited zones in the Hillsborough (East) River in Prince Edward Island, in the Summer of 2014.for (A) the low intensity rain event - at 15 mm over 14 h (B) the high intensity rain event - 40 mm in 14 h.

For instance, the Approved and Restricted zones had higher oocyst counts than Prohibited zones, and the Prohibited zone in our study (Fig. 1) is the point of entry to Northumberland Strait, which receives the greatest impact from tidal flow.

Water temperatures in the range of 8-29° C and high salinity levels (23–32 ppt) have been suggested as having a negative effect on the survival of *Cryptosporidium* spp. (Francavilla et al., 2012). Although we did not measure water temperature and salinity in this study, historical values suggest that during the summer time in the Hillsborough River, temperature and salinity can reach the above values, which could impact our ability to detect this pathogen.

It is well established that rainfall-associated run-off from agricultural and urban land, and wastewater discharges into rivers, estuaries, and coastal waters are associated with seawater and shellfish contamination (Campos and Lees, 2014; Coulliette and Noble, 2008; Kirby-Smith and White, 2006; Riou et al., 2007). However, there is limited discussion in the literature of protozoan contamination associated with rainfall events (Gómez-Couso, 2003; Willis et al., 2013). In our study, Restricted zones were located downstream from Approved zones (Fig. 1) and surrounded by more agricultural land (data not shown) than the Prohibited zone, where the main urban area and harbour of PEI is located. This difference in land use might also explain the higher oocyst concentrations were found in Restricted zones. However, the low sedimentation rate of *Cryptosporidium* spp. oocysts (Rose et al., 1997) and environmental conditions present in these area (e.g. tidal flow) make it difficult to assess the effect of land use on water contamination (Graczyk et al., 2000).

In Prince Edward Island, bay closure times and exposure zones are based on the coliform counts (CSSP, 2008; Suavé, 2010). However, closures times based on fecal coliform contamination have been poorly correlated with *Cryptosporidium* spp. oocyst counts (Gómez-Couso, 2003). A significant number of viable oocysts haven been found after the end of closure times, based on coliform counts (Gómez-Couso, 2003). Willis et al. (2014) found that under experimental conditions of constant exposure of oysters to *Cryptosporidium* spp. oocysts, oysters were still eliminating *C. parvum* oocysts at seven days post-transfer to a clean tank. This suggests that after seven days oysters are still contaminated with infective oocysts and could potentially be a source of infection to the human population.

Although we observed low levels of contamination in this study, they could reach levels above the median infectious doses of 10 oocysts, especially if several contaminated oysters were consumed at one time. While the data generated from this study are limited, they can still provide baseline information for the development of risk assessment of human exposure to this parasite by the consumption of raw oysters harvested from these areas in PEI.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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